Reciprocal effects in human hemoglobin: Direct measurement of the dimer–tetramer association constant at partial oxygen saturation

(linkage/allosteric proteins/thermodynamics)

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ABSTRACT   An equilibrium gel permeation technique has been developed for determining as a function of oxygenation state the equilibrium constants for association of hemoglobin subunits. By using this method, the dimer–tetramer constant for human hemoglobin at a partial oxygenation state corresponding to 20% saturation for tetramers has been determined as 3.7×10^6 M^{-1} (dimers). Under the same conditions the corresponding constant for fully oxygenated hemoglobin is 4.1×10^5 M^{-1}. These results are found to be in good agreement with the predicted behavior of the association reaction based upon oxygen binding curves measured as a function of protein concentration. Thus a high degree of consistency is found between the two independent experimental approaches to the reciprocal effects of this linkage system, lending support to the theory proposed earlier for these phenomena.

The linkage between oxygen binding and dimer–tetramer association in human hemoglobin has been firmly established by experimental demonstration of concentration dependence in ligand-binding curves measured in dilute solutions (1–3) and by the finding of widely different dimer–tetramer association constants for liganded and unliganded hemoglobins (4–6). However, the dimer–tetramer association constant at conditions of partial saturation with oxygen has not been determined experimentally. We report here such a determination.

The changes in subunit interactions that accompany oxygen binding may, in principle, be obtained from a knowledge of the equilibrium constants for dissociation of oligomeric hemoglobins into constituent subunits as a function of oxygenation state (7–11). Thus, apart from the novelty of demonstrating this reciprocal effect in human hemoglobin, generation of the capability to measure such quantities opens up the possibility of their use, long predicted on theoretical grounds, in real experimental systems. In human hemoglobin a direct determination of the dimer–tetramer equilibrium constant is technically very difficult due to the necessity of controlling precisely the oxygenation state during measurements of subunit dissociation while minimizing oxidation, and also due to the extremely high values (e.g., 10^{11} M^{-1}) that may be assumed by this constant (1, 5, 6). Expressed as an association constant, the quantity of interest is:

$$^xK_2 = \frac{[\text{Total concentration of tetramers}]}{[\text{Total concentration of dimers}]^2}$$
$$= \frac{{}^0K_2 [1 + K_{41}(X) + K_{42}(X)^2 + K_{43}(X)^3 + K_{44}(X)^4]}{[1 + K_{21}(X) + K_{22}(X)^2]^2}, \quad [1]$$

in which (X) is molar concentration of oxygen, K_{ni} are the Adair binding constants for tetrameric ($n = 4$) and dimeric ($n = 2$) species in various states of ligation, i, and ${}^0K_2$ is the dimer–tetramer association constant for unliganded hemoglobin (see ref. 10 for derivation of these relationships). Knowledge of these

seven constants permits an estimate of the changes in intersubunit contact energies that accompany oxygenation of tetrameric hemoglobin (3, 10, 11). With sufficiently accurate data an experimental determination of $^xK_2$ vs. (X) would, in principle, provide such information. In practice no determination of $^xK_2$ at partial saturation has been reported prior to the present study.

An alternative method for estimating the seven constituent constants of the right side of Eq. 1 lies in determining oxygen-binding curves as a function of hemoglobin concentration and combining these data with independent estimates of some of the constants. This approach has been found to be experimentally feasible with human hemoglobin (3, 5, 11). From the set of equilibrium constants obtained by this approach the expected value of $^xK_2$ corresponding to any particular value of (X) may be predicted. Thus the determination of $^xK_2$ corresponding to a well-defined state of partial saturation would provide a highly valuable independent check on the validity of such results and would by itself indicate the general manner in which the intersubunit constraints are altered during partial oxygenation (10). In this paper we describe such a determination based upon measurements with human hemoglobin under atmospherically induced conditions of total deoxygenation, 20% saturation, and full oxygenation. The results are compared with predictions from the study of concentration-dependent oxygenation curves carried out under the same conditions (3).

EXPERIMENTAL METHODS

Measurements of the average degree of aggregation in hemoglobin solutions were carried out by using the equilibrium gel permeation technique described previously (12). At each concentration (and under a given oxygenation state) the degree of aggregation is reflected in the experimentally measured *partition cross-section* $\bar{\xi}_w$ representing the average cross-sectional area accessible to solute within a small gel-packed flow cell. For the dimer–tetramer equilibrium:

$$\bar{\xi}_w = \xi_T + f_D(\xi_D - \xi_T), \quad [2]$$

in which ξ_D and ξ_T are the species partition cross-sections and f_D is the weight fraction dimer at equilibrium solute concentration C_T. Direct optical determination of $\bar{\xi}_w$ is carried out at a series of total solute concentrations, C_T, generating a dissociation curve ($\bar{\xi}_w$ vs. C_T) as the average aggregation state is shifted according to the law of mass action. The equilibrium constant $^xK_2$ is then calculated from

$$^xK_2 = \frac{(1 - f_D)}{f_D{}^2 C_T}. \quad [3]$$

The dissociation curves are analyzed in terms of Eqs. 2 and 3

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by using least-squares methods for the species cross-sections (ξ_D, ξ_T) and the equilibrium constant $^xK_2$ (13).

Instrumentation. The instrument used for these measurements was a computer-controlled single-photon-counting spectrophotometer described previously (12). Modifications carried out for these studies consisted of replacing the flow system and measurement cells with an all-glass flow train and an oxygen sensor as diagrammed in Fig. 1. The system permits alternate passage through the measurement cells of samples from either the buffer or protein solution reservoirs. These reservoirs were simultaneously equilibrated with the same gas atmosphere from a tank of fixed composition—i.e., a predetermined oxygen/nitrogen mixture (Matheson) of precisely known (1% relative to O_2 value) composition—or with pure gas (N_2 or O_2). By appropriate adjustment of valves the flow train also permits passage of buffer solution from the buffer reservoir into the solution reservoir. Thus the sample can be diluted after determination of $\bar{\xi}_w$ while equilibration with a given gas atmosphere is maintained. The sample cells are subsequently saturated with the diluted solution and a new value of $\bar{\xi}_w$ is determined. The precise dilution, and new value of C_T, are determined at each step by the absorbances measured in the reference cell. By repeating this procedure at a series of dilutions a complete dissociation curve ($\bar{\xi}_w$ vs. C_T) is generated.

The oxygen sensor at the end of the flow train utilized a Beckman electrode (no. 39065) with polarizing voltage source and amplifier constructed in this laboratory. Two measurement cells were incorporated into the system: A gel-packed quartz flow cell (0.2 ml bed volume, 0.5 cm pathlength) containing Sephadex G-100 (Pharmacia lot 6164) in series with a similar reference flow cell containing no gel. Tygon connectors to these cells comprised the only nonglass parts. Their joints were sealed with glyptal (General Electric). Experiments were carried out at 21.5°C ± 0.2°C. Absorbance measurements were made at wavelengths of 220, 230, 240, and 415 nm. The sample compartment was purged with dry nitrogen during all experiments. Linearity of the photometric system according to Beer's law (up to absorbances of 4.0) was verified at each wavelength by using weighed dilutions of myoglobin and oxyhemoglobin solutions. Temperature of the cell compartment was regulated by means of an external air bath. Temperature of the flow cells was additionally controlled by means of a water-thermostated cell carriage.

Materials. Hemoglobin A was purified from freshly drawn blood according to the method of Williams and Tsay (14).

Hemoglobin Hirose (15) was a gift from Austen F. Riggs (University of Texas, Austin, TX). Protein samples were stored in liquid nitrogen prior to their use. Buffer used in all experiments consisted of 0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA, pH 7.4. Sephadex G-100 was obtained from Pharmacia Fine Chemicals. Glyptal red paint for anaerobic sealing of tubing joints was obtained from General Electric.

Calibration of the System. Response of the oxygen sensor was determined for buffer solutions equilibrated against a series of tanks containing fixed mixtures of oxygen and nitrogen (Matheson) ranging between 0.35 torr (1 torr = 133 pascals) (0.05% O_2) and 7.60 torr (1% O_2). The readings obtained were correlated with spectral determinations obtained on hemoglobin solutions within the reference flow cell. The spectral determinations were carried out by making point-by-point absorbance measurements at intervals of 5 nm between 400 nm and 500 nm. The spectra were analyzed for percentages of oxy-, deoxy-, and methemoglobin, according to a nonlinear least-squares procedure developed by B. Turner in this laboratory. For end point (deoxy) verification, hemoglobin solutions containing 0.1% sodium dithionite were used.

Determination of Concentration-Dependent Partition Cross-Sections for Hemoglobin. Experiments with human hemoglobin A were carried out under four sets of conditions: fully oxygenated (i.e., under 1 atmosphere, 155 torr oxygen partial pressure), fully deoxygenated under equilibration with pure nitrogen, under nitrogen in the presence of 0.1% sodium dithionite, and under 0.425% oxygen (i.e., 3.23 torr oxygen partial pressure). Under each set of conditions experiments were carried out at different hemoglobin concentrations, within the maximum range 0.1 μM to 60.0 μM in order to define dissociation curves under each condition. Measurements were similarly carried out for hemoglobin Hirose (15) under fully oxygenated conditions.

RESULTS

Verification of Anaerobic State within the Flow Train. Results of the oxygen sensor calibration are shown in Fig. 2. It can be seen that a highly linear relationship exists between the oxygen sensor output and oxygen concentrations. Extrapolation of the values obtained for the O_2/N_2 mixtures to zero oxygen concentrations was found to be in excellent agreement with the pure nitrogen end point and in good agreement with the end point determined in the presence of dithionite. Least squares analysis of the spectra determined for the deoxygenated solu-
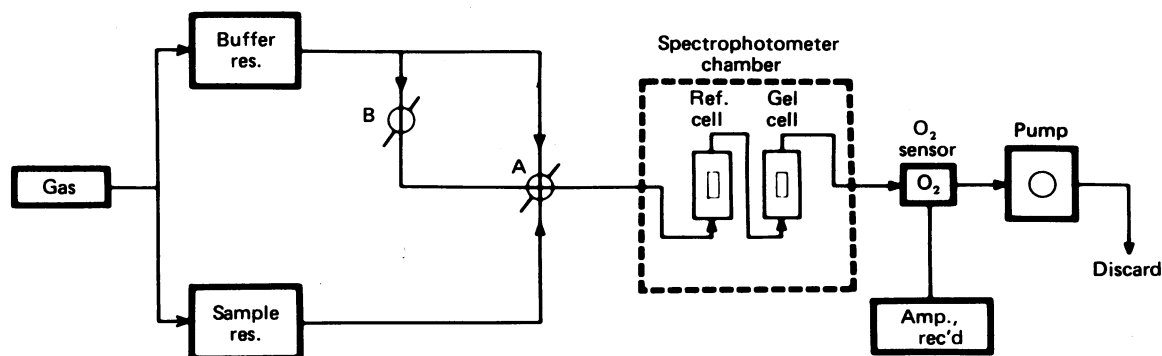

FIG. 1.   Flow diagram of modified photon-counting system used in this study. Buffer and sample reservoirs are a 1-liter aspirator bottle and a 50-ml erlenmeyer flask, respectively. The flow train is made of 2-mm-bore glass tubing with movable joints of Tygon tubing painted with glyptal red (General Electric). Stopcocks A and B are four-way and two-way valves, respectively, whose various combinations will allow passage of buffer or sample into the flow cells and permit filling the sample reservoir with buffer as necessary while maintaining a constant atmosphere in the flow train during manipulations. The spectrophotometer and sample chamber comprise the photon-counting instrument as previously described (12). The oxygen sensor is housed in a 1 × 1 × 2 inch (1 inch = 2.54 cm) Lucite block with a 2-mm bore passage for effluent solution. Output from the amplifier is recorded vs. time on a Sargent–Welch recorder. The quartz flow cells are equipped with ground glass fittings and sealed with Corning vacuum grease to ensure an air-tight seal. A flow rate of 6.0 ml/hr was maintained by an LKB peristaltic pump.
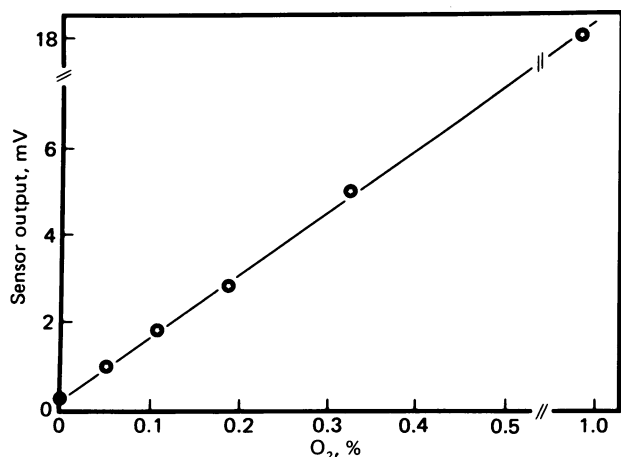
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FIG. 2. Oxygen sensor calibration curve (see text). Symbols with stars indicate oxygen sensor output vs. percent oxygen from individual N_2/O_2 mixtures of predetermined composition. The zero oxygen point (solid symbol) represents the end point obtained by using a pure nitrogen tank in both the presence and absence of 0.1% sodium dithionite.

tions (2 μM heme) indicated the dithionite-containing solution to be 100% ± 0.2% unliganded; the nitrogen-purged solution (no dithionite) contained an estimated 2.3% ± 2.5% (65% confidence limits) oxyhemoglobin, and the oxygenated solution was estimated to contain 97.3% ± 2% oxyhemoglobin. These two dithionite-free solutions contained an estimated 2–3% methemoglobin. These estimates derived from least-squares analysis provide uncertainty limits defining the maximum amount of oxygenated hemoglobin present under the atmospherically induced anaerobic conditions of the flow train. However, these spectral results, requiring simultaneous analysis for three species, do not provide highly accurate estimates. The percentage of oxygenated hemoglobin under anaerobic conditions is probably much less than the estimated 2.3% because (*i*) this value is itself smaller than the 65% confidence limits defined by the data, and (*ii*) the electrode calibration results and subsequent readings taken routinely during experiments
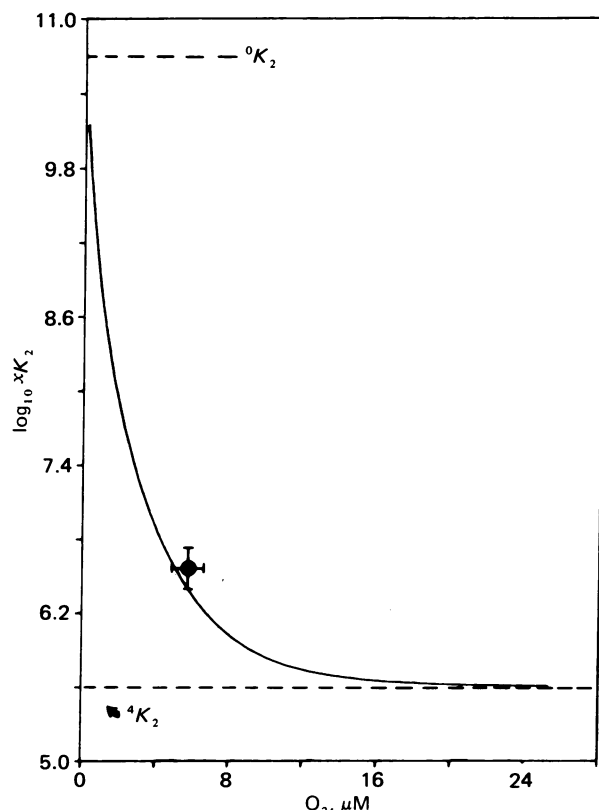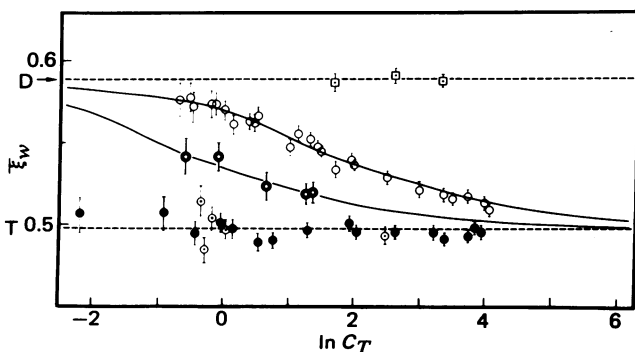

FIG. 3. Weight-average partition cross-sections $\bar{\xi}_w$ vs. total protein concentrations C_T (μM heme). The range of concentration was 0.1–60 μM heme. □, Data from oxygenated hemoglobin Hirose, which serves as a dimer end-point estimate: $\bar{\xi}_D$ = 0.5888 ± 0.0029. ○, Oxyhemoglobin; ⊙, hemoglobin deoxygenated under a N_2 atmosphere only; ●, deoxyhemoglobin with 0.1% dithionite. These latter two sets define the tetramer endpoint $\bar{\xi}_T$ = 0.4979 ± 0.0068. Circles with stars are for hemoglobin at a partial pressure of 0.425 ± 0.05% oxygen (3.23 ± 0.4 torr) corresponding to 20% oxygenation of hemoglobin tetramers. Solid curves represent the best least-squares fit of each data set to a dimer–tetramer association reaction. Experiments were carried out in 0.1 M Tris·HCl/0.1 M NaCl/1 mM Na_2EDTA, pH 7.40, 21.5°C.

FIG. 4. Effect of oxygen on the dimer–tetramer association constant for human hemoglobin. $^0K_2$ is the constant for unliganded hemoglobin determined previously (6). $^4K_2$ is the constant for fully oxygenated hemoglobin determined in this study. Broken horizontal lines represent values of the logarithms of these constants. The solid point represents the dimer–tetramer constant determined at a partial pressure of 3.23 torr. The solid line is the predicted variation of the association constant based upon binding constants from oxygenation curves measured as a function of hemoglobin concentration (3) and the value of $^4K_2$ determined in this study. The Henry's law constant for these conditions is 1.77 μmol O_2/torr.

had a much smaller uncertainty, indicating the presence of not more than 0.3% oxygenated hemoglobin in the nitrogen-purged solutions. Both the spectral and polarographic methods indicated constancy of better than 0.2% during a given experiment.

Dissociation of Hemoglobin A. Weight-average partition cross-sections determined as a function of hemoglobin concentration are shown in Fig. 3. Broken lines indicate the estimated cross-sections for dimers and tetramers based upon calibration of the gel for molecular radius (16) and upon least squares analysis for end-point determinations (13). It may be seen that the partition cross-sections for hemoglobin Hirose at various concentrations fall consistently on the line for cross-section of dimer, consistent with the finding that this variant exists essentially in the dimeric state under oxygenated conditions (15). Values of partition cross-sections for unliganded hemoglobin are seen to fall very near the line representing tetrameric hemoglobin, for both the dithionite-free and the dithionite-containing solutions. This result is entirely consistent with predicted behavior over this concentration range based upon the kinetic determination of the equilibrium constant pertaining to these conditions: 5.1 × 10^{10} M^{-1} (6).

Data obtained for the oxygenated solutions show the expected variation for hemoglobin A_O and provide a dissociation curve from which the equilibrium constant may be estimated. Least-squares analysis of these data yielded a value of 4.02 ×

10^5 M^{-1} dimer ($\Delta G = -7.56 \pm 0.27$ kcal/mol of dimer) (1 kcal = 4.18 kJ). This value is in reasonable agreement with previous determinations made under these conditions (6).

Data obtained under a partial pressure of $0.425 \pm 0.05\%$ oxygen (3.23 ± 0.4 torr, 5.87 μM O_2) are also shown in Fig. 3. At this partial pressure, hemoglobin tetramers are 20% saturated as determined under identical conditions (10). The solid lines shown for data of both the partially and fully oxygenated hemoglobins represent the least-squares fit of data points to Eqs. 2 and 3. The best fit molar association constant $^xK_2$ from the partially oxygenated hemoglobin data was found to be 3.66 × 10^6 M^{-1} (dimers); the standard free energy of tetramer formation is -8.84 ± 0.2 kcal/mol of dimer. This value of $^xK_2$ is plotted on Fig. 4 along with the predicted variation with oxygen concentration of this function (Eq. 1) based upon the values of binding constants derived from oxygenation curves (10), the kinetically determined association constant for unliganded hemoglobin (5, 6), and the oxyhemoglobin association constant determined in this study.

DISCUSSION

Development of a method for reliable measurement of hemoglobin self-association constants under equilibration with precisely controlled gaseous atmospheres and in the absence of excessive oxidation has proved exceedingly difficult. The modest results presented in this communication have required several years of continuous effort. In the final configuration employed in these studies our system permits dissociation curves to be measured down to concentrations of 0.1 μM heme (1.6 μg/ml). The oxygenation state may be controlled to within narrow limits (e.g., 0.3%), whereas the estimation of methemoglobin is subject to an uncertainty of approximately ten times this magnitude. We have found that this degree of control was not achievable using conventional gel chromatography techniques, whereas the use of small gel-packed flow cells instead of chromatographic columns is of singular value in these studies.

For human hemoglobin the value of $^xK_2$ determined at 20% saturation with oxygen, as compared with the value $^4K_2$ at 100% saturation, is seen (Fig. 4) to vary in accordance with the predicted curve derived from oxygenation studies under the same buffer conditions and temperature. Thus a high degree of consistency is found between the two independent experimental approaches to this linkage system, and greater confidence can be placed upon the linkage scheme proposed to account for these phenomena (10). Although detailed resolution of the constituent constants in Eq. 1 cannot of course be carried out from the two values of $^xK_2$ determined in this study, the general nature of the oxygen-linked energetic transitions in hemoglobin is clearly indicated. From simulations on the behavior of the function $^xK_2$ vs. (X) it has been found (10) that energy distributions in which approximately half the intersubunit contact energy is altered with the first binding step have the characteristic shape of the curve in Fig. 4 and that models in which substantially less than half the energy is altered are easily distinguished by their much gentler slope. The present results thus provide an important cross-check on the validity of the energy distribution derived from the oxygenation studies (3) as to the general conclusions drawn. Because each approach must be subject to different kinds of systematic errors, and re-

sults of each are highly sensitive to such errors, it is unlikely that the agreement is coincidental.

Results of this study have important implications for determinations of oxygenation curves of human hemoglobin in dilute solution, because they point most directly to the necessity of correcting such curves for the effects of dimers (2, 17). On the basis of the values of $^xK_2$ measured in this study we may calculate that, in going from fully saturated to 20% saturated conditions, the percentage of dimers present at 60 μM heme (where many oxygen-binding measurements have been reported) changes from 13.4% to 4.7%. Effects of these dimers on the apparent Adair constants obtained by ignoring them in the analysis of oxygen-binding curves have been described in detail elsewhere (17).

Finally, it should be noted that the method described here may be usefully applied to the study of a variety of hemoglobin systems, particularly those with high oxygen affinity and high order of polymerization. A study on self-association of isolated hemoglobin β chains as a function of oxygenation state has been carried out with this technique (18, 19). In that system the linkage between oxygenation and monomer–tetramer association is most easily established from dissociation measurements rather than from oxygenation studies.

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